The immunologic role of thymectomy in the treatment of myasthenia gravis: Implication of thymus-associated B-lymphocyte subset in reduction of the anti-acetylcholine receptor antibody titer

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Background and Purpose: Thymectomy is generally accepted as the major option of treatment for myasthenia gravis. To elucidate the biological role of thymectomy in the treatment of myasthenia gravis, the immunologic characteristics of the thymus was studied in association with the postoperative kinetics of the anti-acetylcholine receptor antibody titer.

Materials and Methods: Thirty-four patients with nonthymomatous myasthenia gravis who had positive anti-acetylcholine receptor antibody titer and undergoing extended thymectomy were subjected to the study. Reduction of anti-acetylcholine receptor antibody titer was evaluated in terms of the proportion of anti-acetylcholine receptor antibody titer at 1 year after thymectomy to that before the operation. The numbers of B lymphocytes ($CD19^+$ cells) and the germinal center B lymphocytes ($CD19^+CD38^{high}$ cells) present in 1 g of the thymic tissue were calculated by flow cytometry.

Results: The proportion of anti-acetylcholine receptor antibody titer at 1 year after thymectomy ranged from 27.5% to 150%. The numbers of B lymphocytes and the germinal center B lymphocytes in 1 g of the thymic tissue ranged from 0.19×10^6/g to 162.8×10^6/g and from 0.09×10^6/g to 33.4×10^6/g, respectively. The proportion of anti-acetylcholine receptor antibody titer at 1 year after thymectomy had a significant inverted correlation with the number of B lymphocytes ($P = .002$) as well as that of the germinal center B lymphocytes ($P = .007$).

Conclusion: Effectiveness of thymectomy was dependent on predominance of B lymphocytes and the germinal center B lymphocytes in the thymus, suggesting that one of the biological roles of thymectomy in the treatment of myasthenia gravis is removing the thymus-associated germinal centers.

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Myasthenia gravis (MG) is an autoimmune disease characterized by fatigable muscle weakness. Nicotinic acetylcholine receptor (AchR) is the major autoantigen in MG, and production of anti-nicotinic acetylcholine receptor antibody (anti-AchR Ab) is supposed to cause reduction of the number of AchR at the neuromuscular junction, resulting in muscle weakness.[1-3]

MG is widely known for its frequent association with disorders of the thymus including hyperplasia and thymoma.[4-6] Thymectomy has been shown to result in remission or reduction of MG symptoms in nearly 90% of patients and is generally accepted as a major option for treatment,[7-9] although the operative procedure is still controversial.[10-12] Consistent with improvement of the clinical symptoms, the anti-AchR Ab titer was shown to decline after thymectomy.[13,14]

The typical microscopic appearance in patients with nonthymomatous MG is follicular hyperplasia characterized by the prominent germinal centers in the medulla.[4,5] Although the thymus is the central organ for T-lymphocyte differentiation, the germinal center is where B lymphocytes differentiate to the cells producing antibodies with high affinity to the antigens. Several lines of in vitro studies revealed the anti-AchR Ab production by the lymphocytes recovered from the MG thymus,[13,15] suggesting the critical role of the thymic germinal centers in the pathogenesis of MG. However, the clinical implication of thymus-associated germinal centers in the MG thymus remain to be clarified.

In the present study, to reveal the clinical significance of the thymus-associated B lymphocytes in the treatment of MG, we performed quantitative evaluation of the lymphocyte subset in the thymus, in conjunction with transition of the anti-AchR Ab titer after thymectomy.

Methods

Patients

There were 97 patients with MG who underwent surgical treatment at Osaka University Hospital between l991 and 2000. The diagnosis was done by neurologists on the basis of the clinical symptoms, findings of electromyogram, response to edrophonium (Tensilon test), and measurement of the anti-AchR Ab titer in the serum. Other neurologic disorders were critically excluded.

To focus on the immunologic implication of thymectomy in patients with nonthymomatous MG in this study, 26 patients with thymomatous MG were excluded. Among the remaining 71 nonthymomatous patients, 34 patients who met the following criteria were subjected to the present study: (1) onset of the disease was after 10 years of age; (2) the serum anti-AchR Ab titer was positive (so-called "seropositive"); (3) removal of the thymus and the adjacent fatty tissue (extended thymectomy) was performed; (4) oral daily dose of more than 10 mg of prednisone or intravenous high-dose steroid therapy was not administrated before thymectomy; (5) postoperative steroid therapy was not added or postoperative oral daily dose of prednisone was not increased before final evaluation of the anti-AchR Ab titer at 1 year after thymectomy.

Osserman's classification was type I in 3 patients, type IIA in 11 patients, and type IIB in 20 patients. The patients were informed of the nature of the study and had given consent to the use of the materials prior to the operation.

Evaluation of Reduction in Serum Anti-AchR Antibody Titer

Anti-AchR Ab titer was measured by the method of radioimmunoassay at Shionogi Laboratory (Osaka, Japan) as previously described.[15] The reduction of anti-AchR Ab titer was evaluated at 1 year after thymectomy. The proportion of the titer (% Ab) at 1 year after thymectomy to the preoperative value was calculated.

Histologic Evaluation of the Thymus

Several portions of the resected thymus were subjected to the routine pathologic examination. Hematoxylin and eosin–stained sections were prepared from paraffin-embedded blocks and microscopically examined under low magnification (×40). The average number of the germinal centers identified in 1 visual field was evaluated by counting in 3 to 4 randomly selected visual fields of at least 2 sections of the resected thymus.

Recovery of Lymphocytes from the Thymus and Flow Cytometric Analysis

The remaining thymic tissue was weighed, minced with scissors, pressed against a stainless steel mesh, and the lymphocytes freshly isolated, as reported previously.[16,17] The recovered lymphocytes were suspended in cold phosphate-buffered saline solution (PBS) as a single-cell suspension and counted. The total number of the recovered lymphocytes was divided by the weight of the specimen, and the number of lymphocytes derived from 1 g of the thymic tissue was obtained. When a considerable number of erythrocytes were present in the cell suspension, erythrocytes were removed by a Ficoll-Hypaque (Lymphoprep, Nycomed, Oslo, Norway) density gradient. Finally, 1×10^7 lymphocytes were suspended in 1 mL of cold PBS before subjected to the flow cytometric study.

Flow Cytometric Analysis of Surface Marker Expression of the Lymphocytes and Quantitative Evaluation of B-Lymphocyte Subset

Fluorescein isothiocyanate (FITC)-conjugated anti-human CD19 (a marker of B lymphocytes) and phycoerythrin (PE)-conjugated anti-human CD38 (a marker of B lymphocytes in the germinal center) were purchased from DAKO (Glostrup, Denmark) and Becton-Dickinson (San Jose, Calif), respectively. FITC-conjugated control mouse immunoglobulin G (IgG) and PE-conjugated control mouse IgG were also purchased from Becton-Dickinson.

Surface marker expression was evaluated by flow cytometric study following the method reported previously.[16-18] In brief, 1×10^6 lymphocytes were labeled by FITC-conjugated anti-CD19 antibody on ice for 20 minutes, followed by 2 washes in cold PBS. At least 10,000 events were acquired to evaluate the surface marker expression with the use of a FACScan cytometer (Becton-Dickinson), and the data were analyzed by the program Lysis or Cell Quest (Becton-Dickinson). The negative level of expression was determined according to the control mouse IgG staining. The number of B lymphocytes was calculated by the number of lymphocytes in 1 g of the thymic tissue and the proportion of $CD19^+$ cells.

When the germinal center B lymphocytes were being evaluated, PE-conjugated anti-human CD38 was also added to the cell suspension, and the simultaneous analysis of CD19 and CD38 expressions by 2-color flow cytometric method was done. The germinal center B lymphocytes were defined as $CD19^+$ cells expressing CD38 at a high level. The number of germinal center B lymphocytes was also calculated by the number of lymphocytes in 1 g of the thymic tissue and the proportion of $CD19^+CD38^{high}$ cells.

Simultaneous analysis of CD19 and CD38 expressions was done in 17 patients. In the remaining 17 patients, CD19 expression alone was evaluated.

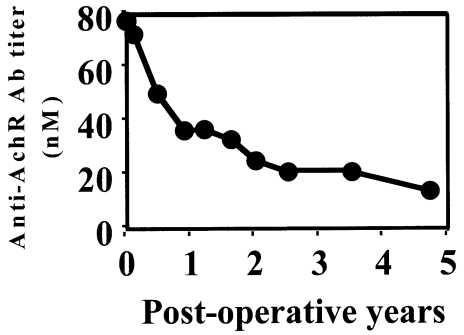

Figure 1. The typical transition of anti-AchR Ab titer after thymectomy.

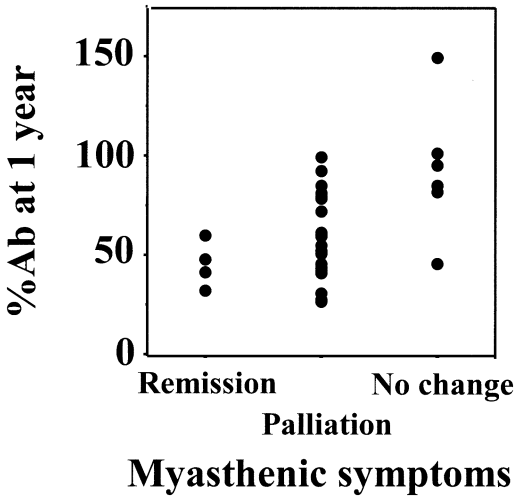

Figure 2. The clinical outcome and the reduction of anti-AchR Ab titer.

Statistical Analysis

Correlation between 2 variables was calculated by the Pearson correlation coefficient. Statistical analyses were done with the SPSS software (SPSS, Inc, Chicago, Ill).

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Results

Relationship between the Clinical Outcome and the Reduction of Anti-AchR Ab Titer

Remission of the myasthenic symptoms was obtained in 4 patients (11.8%), and palliation was obtained in 24 patients (70.6%) at 1 year after thymectomy. The myasthenic symptoms were unchanged and thymectomy was not considered as effective in the remaining 6 patients (17.6%).

The typical transition of anti-AchR Ab titer after thymectomy is shown in Figure 1. In this patient, anti-AchR Ab titer decreased slowly during 5 years, and %Ab at 1 year was 47.1%. Among all patients, %Ab at 1 year ranged from 27.5% to 150%.

Figure 2 shows % Ab at 1 year in each patient of the groups with remission, palliation, or no change. The mean % Ab at 1 year was 45.9%, 59.7%, and 93.6% in each group, respectively; % Ab at 1 year was reduced to less than 60% in 3 of 4 patients (75%) with remission, in 13 of 24 patients (54.2%) with palliation, and in 1 of 6 patients (16.7%) whose symptoms were unchanged. Thus, reduction of anti-AchR Ab titer reflected the clinical improvement of MG.

Relationship between the Clinical Outcome and the B-Lymphocyte Subset in the Thymus

The number of the lymphocytes recovered from 1 g of the thymic tissue ranged from 2.9×10^6/g to 742×10^6/g.

The representative result of 2-color flow cytometric analysis on CD19 and CD38 expressions is shown in Figure 3. Lymphocytes were gated according to the forward and the side scatter pattern (Figure 3, *A*). The proportion of $CD19^+$ cells was 55% (Figure 3, *C*). $CD38^{high}$ cells comprised 20.5% of $CD19^+$ cells (Figure 3, *D*), and thus the proportion of $CD19^+CD38^{high}$ cells was 11.3%. The number of lymphocytes recovered from the thymic tissue was 294.4×10^6/g in this patient, and therefore the numbers of $CD19^+$ cells (B lymphocytes) and $CD19^+CD38^{high}$ cells (the germinal center B lymphocytes) present in 1 g of the thymic tissue was calculated to be 162.8×10^6/g and 33.4×10^6/g, respectively.

The proportion of $CD19^+$ cells ranged from 1.4% to 56.4%. The number of $CD19^+$ cells in 1 g of the thymic tissue ranged widely from 0.19×10^6/g to 162.8×10^6/g. As shown in Figure 4, % Ab at 1 year had a significant inverted correlation with the number of $CD19^+$ cells (B lymphocytes) in 1 g of the thymic tissue.

The proportion of $CD38^{high}$ cells in the $CD19^+$ population ranged from 1.2% to 20.5%. The number of $CD19^+CD38^{high}$ cells in 1 g of the thymus tissue ranged widely from 0.09×10^6/g to 33.4×10^6/g. As shown in Figure 5, % Ab at 1 year had a significant inverted correlation with the number of $CD19^+CD38^{high}$ cells (germinal center B lymphocytes) in 1 g of the thymic tissue.

Correlation between Flow Cytometric Analysis of B-Lymphocyte Subset and the Microscopic Appearance of the Thymus

In 17 cases where expression of both CD19 and CD38 was simultaneously evaluated, microscopic appearance of the thymus was then examined. Patients were divided into 3 groups according to the number of the germinal centers identified microscopically: below 1 (group A), 1 to 2 (group B), and more than 2 (group C). As shown in Figure 6, the number of germinal center B lymphocytes identified as $CD19^+CD38^{high}$ cells by flow cytometry reflected the abundance of the germinal center in the microscopic appearance.

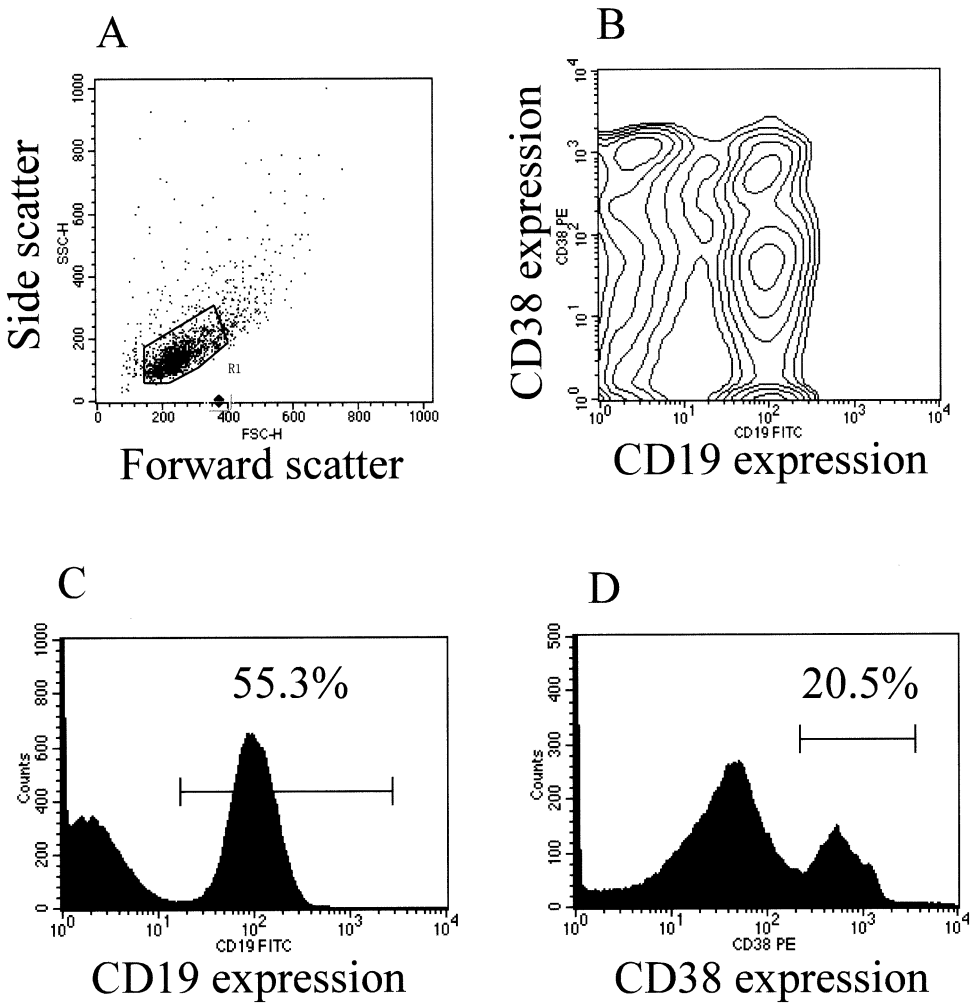

Figure 3. The representative result of flow cytometric analysis. A, Lymphocytes are gated according to the forward and the side scatter pattern. B, Two-color flow cytometric analysis of CD19 and CD38 expressions. C, B cells are gated as $CD19^+$ cells. D, CD38 expression of $CD19^+$ cells. Germinal center B cells are defined as $CD19^+$ cells expressing CD38 at a high level.

Discussion

Although thymectomy is generally accepted as 1 major option of treatment for MG, the effectiveness of thymectomy differs from a patient to patient, and there is a minor but considerable population of patients who are not benefited by thymectomy. The microscopic appearance of the thymus also varies greatly from patient to patient. Instead of follicular hyperplasia, normal appearance as well as the involuted thymus are sometimes encountered. The present study quantitatively evaluated the relationship between immunologic characteristics of the MG thymus and the effectiveness of thymectomy and revealed the significance of the thymus-associated B lymphocytes, especially the germinal center B lymphocytes, in the reduction of anti-AchR Ab titer after thymectomy.

Although there have been numerous studies reporting the effectiveness of thymectomy in the treatment of MG, the change in clinical symptoms has been adopted as the parameter to evaluate the effectiveness of thymectomy. It is not easy to quantitatively evaluate the clinical symptoms, however. In addition, the severity of clinical symptoms might be affected by unrelated situations such as infectious diseases and emotional status. To evaluate the severity of the disease in a quantitative and objective manner in each particular patient, we chose the serum anti-AchR Ab titer as the biological variable. As shown in Figure 2, reduction of anti-AchR Ab titer reflected improvement of the clinical symptoms.

Autoantibodies to AchR-associated proteins, but not to AchR itself, have been suggested to be responsible for

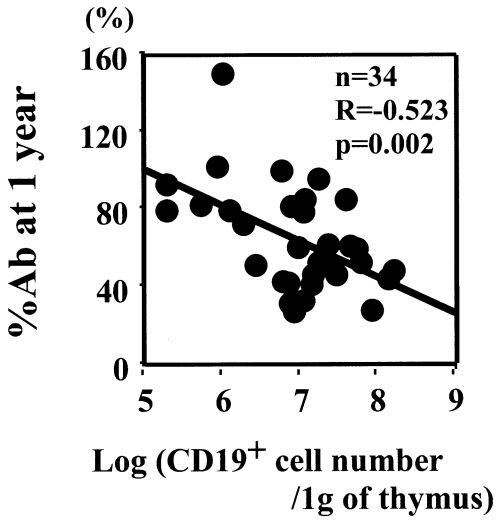

Figure 4. The correlation between % Ab at 1 year and the number of CD19$^+$ cells (B lymphocytes) present in 1 g of the thymic tissue.

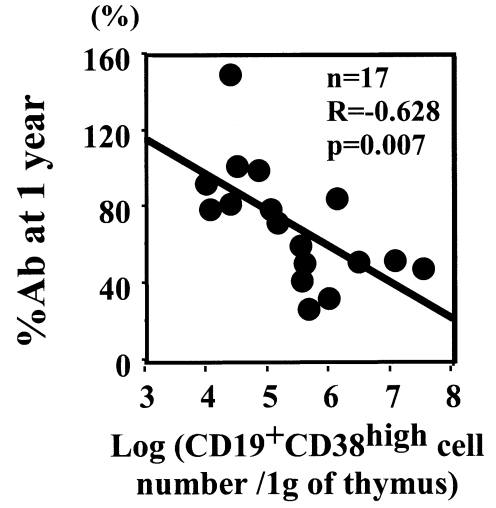

Figure 5. The correlation between % Ab at 1 year and the number of CD19$^+$CD38^{high} cells (the germinal center B lymphocytes) present in 1 g of the thymic tissue.

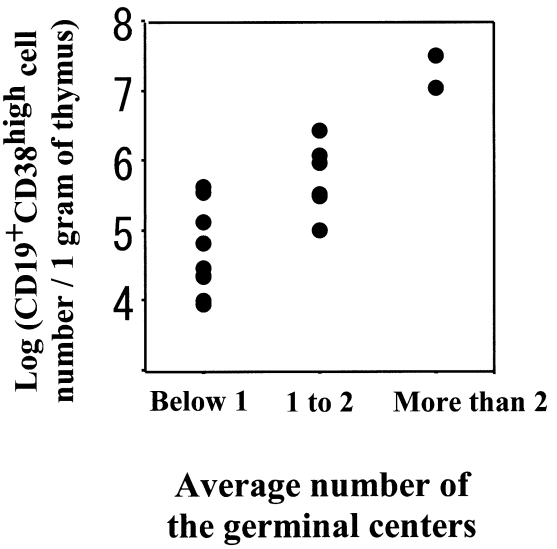

Figure 6. Correlation between the number of CD19$^+$CD38^{high} cells and the number of germinal centers identified microscopically.

muscle weakness of seronegative MG patients.[19] More recently, muscle-specific receptor tyrosine kinase was identified as the autoantigen in the seronegative MG patients,[20,21] indicating that the anti-AchR Ab-negative MG is a disease distinct from the anti-AchR Ab-positive MG. Anti-AchR Ab-negative (seronegative) MG patients, therefore, were excluded from the present study.

Steroid therapy is another major option of treatment for MG, and it is frequently administered prior to thymectomy. Because T lymphocytes in the thymus and B lymphocytes in the germinal center are known to be sensitive to steroid, administration of high-dose steroid possibly affects the lymphocyte population also in the MG thymus. Therefore, the patients who had daily prednisone dose of more than 10 mg or those who experienced preoperative steroid pulse therapy were excluded from the study.

Under the above conditions, based on results obtained from the seropositive and non-thymomatous patients who did not have preoperative high-dose steroid therapy, the present study revealed that reduction of anti-AchR Ab titer after thymectomy was dependent on abundance of B lymphocytes, especially the germinal center B lymphocytes, in the thymus. The present results suggested that thymectomy contributes to elimination of anti-AchR Ab–producing cells in the thymus. Our previous study has shown that the CD4$^+$ T-lymphocyte (helper T-cell) subset in the thymus is correlated with anti-AchR Ab titer.[16] This observation seems compatible with the result in the present study because antibody production requires helper T- and B-lymphocyte interaction and this possibly occurs also in the germinal centers of the thymus in patients with MG. Our previous study, however, revealed the abnormal expression of Bcl-2 protein in the thymic germinal centers of the patients with MG,[22] suggesting the possible selection of autoreactive B lymphocytes in the MG thymus. Further studies remain to be done to fully reveal the mechanisms of development of autoreactive B lymphocytes in the MG thymus.

Although reduction of the myasthenic symptoms was often accompanied by reduction of serum anti-AchR Ab titer, clinical improvement is observed in some cases where serum anti-AchR Ab titer remains unchanged after thymectomy. Contrarily, myasthenic symptoms are not relieved in some cases despite reduction of anti-AchR Ab titer. Several autoantibodies to the skeletal muscle, such as anti-ryanod-

ine receptor antibody[23] and anti-titin antibody,[24] were also identified and shown to be correlated with clinical severity of MG.[25] The serum titers of these antibodies might have some role in the clinical outcome of the patients who had discrepancy in the kinetics of serum anti-AchR Ab titer and clinical outcome. Further study focusing on the role of the thymus in production of these autoantibodies remains to be done.

Because the present study strictly focused on the patients with nonthymomatous MG, the role of thymectomy in thymomatous MG could not be approached. Our recent study showed that type B1 and B2 thymomas, according to the World Health Organization histologic classification system, are significantly more often associated with MG than other types of thymomas.[26,27] Type B thymomas were shown to induce $CD4^+CD8^+$ cells, the T lymphocytes with the phenotype of the cortical thymocytes. The environment presented by the thymoma neoplastic epithelial cells whose human leukocyte antigen-DR expression is down-regulated is supposed to alter the selection mechanisms of $CD4^+CD8^+$ cells and might be responsible for development of autoreactive T lymphocytes and possibly supply these cells to the peripheral organs.[28-34] T-lymphocyte subset, therefore, should be more focused than B-lymphocyte subset in the patients with thymomatous MG.

In conclusion, at least one of the biological roles of thymectomy in the treatment of MG was suggested to be the removal of the thymus-associated germinal centers. To establish the method for preoperative evaluation of the immunologic characteristics of the thymus seems helpful for clinical practices, for example, in selecting the patients with MG who are most benefited by thymectomy.

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Discussion

Dr Steven J. Mentzer *(Boston, Mass).* If I understood this correctly, you think that the supply of the antibody-producing cells originates in the thymus. You believe the delay in resolution of MG is not due to the fact that you have eliminated the entire population of antibody-producing cells, but that you have eliminated the organ producing these cells.

Dr Okumura. The first onset might occur in the peripheral organs, but after the sensitization of the T lymphocytes, those T lymphocytes can move to the thymus and there form germinal centers. The B lymphocytes come peripherally to the germinal centers in the thymus and there develop into the antibody-producing cells with higher affinity antibody. Those cells go out to the periphery again. Therefore, the central site of high-affinity antibody-producing cells in the patients with MG is probably the thymus.

Dr Ross M. Bremner *(Los Angeles, Calif).* Your conclusion was that some of these thymus glands do not have a lot of B cells and the outcome of thymectomy in those patients is not as good as the outcome in those that have a lot of B cells. Can you get that information just from a biopsy or do you need to remove the entire thymic gland?

Dr Okumura. Previously we did those kinds of experiments. We actually biopsied bone marrow cells, lymph nodes, by culturing those cells in vitro, and those B lymphocytes can produce anti-acetylcholine antibodies.